Labeling and Expression of Opioid Receptors Using N-Terminal Fusion of Fluorescent Proteins

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Introduction

The most efficient fluorescent based binding assay requires detection of Förster resonance energy transfer (FRET) or Luminescence Resonance Energy Transfer (LRET) between two matched fluorescent ligands in the former or a chelated lanthanide donor and fluorescent acceptor in the latter, one attached to the receptor and one attached to the ligand. Although there is an abundance of fluorescent based functional assays for G-coupled protein receptors (GPCRs) [1,2], there are few fluorescent based receptor binding assays, the only commercial fluorescent based binding assay contains SNAP tags [3,4], although capable of FRET it requires the expression of a heterologous protein which is subsequently labeled with a fluorescent tag. Many of the GPCRs including the opioid receptors have short *N*-terminal tails, usually 20-50 amino acids. Early attempts to fuse FP to the receptors yielded poor results such that today fusion to the *C*-terminal tail is the preferred method of labeling receptors. There are notable exceptions such as class II GPCRs that naturally have large extracellular domains such as vasoactive intestinal peptide receptor (VPAC1), these receptors readily accept fusion of a FP to the *N*-terminal tail [5]. GPCRs with large extracellular domains usually have a cleavable signal peptide that enables cell surface expression. Increasing receptor expression at the cell surface by inserting a cleavable signal sequence to heterologous receptors was first described for adrenergic receptors [6] and is now a widely used method for receptor expression. Early studies described for the mu opioid receptor (MOR) with a FP fused to the *N*-terminal of the receptor by insertion of the FP between the signal sequence of the insect endogenously expressed Immunoglobulin Heavy Chain Binding Protein (BiP) and MOR. Cell surface expression was achieved but FP-MOR was limited to insect cells and receptors were observed to be trapped within intracellular compartments [7]. In mammalian cells it appeared that insertion of a signal sequence interfered with the natural regulation of expression via glycosylation [8,9] or alternate strategies such as binding to p24A through the acidic residues at the second extracellular loop [10].

Results and Discussion

Cloning: The nucleotide sequence for GFP was inserted into a plasmid (pN1) already containing mouse MOR (Scheme 1). An EcoRI site was first created in the initial *N*-terminal sequence (MKTIALSYIFCLEF) by replacing the valine (V) by glutamic acid (E) to generate GAATTC and a HindIII site within the receptor sequence ISDCSDPLAPA replacing the amino acids aspartic acid and proline (DP) with glutamic acid and serine (ES) to generate AAGCTT using site directed mutagenesis. GFP nucleotide sequence with the two flanking restriction sites was generated by PCR, using a forward primer with a EcoRI site and a reverse primer with a HindIII site. The PCR product was inserted into the plasmid. Our signal peptide was inserted using nested PCR reactions with a BamHI site engineered into the MOR receptor sequence and a BglII site located in the multiple cloning site of pN1. The human clones of DOR, KOR

Scheme 1. Molecular cloning strategy for production of fluorescent labeled receptor at the N-terminus with custom signal peptide.

and OXR2 fusions were generated by exploiting the BsrGI site on the 3' of the GFP sequence and NotI in the pN1 vector (an internal NotI site in DOR was first removed). Further optimization of the signal sequence was achieved using site directed mutagenesis (proprietary information).

MOR binding assay: Rat cortices were homogenized using 50 mM Tris, pH 7.4, and centrifuged at 16,500 rpm for 10 min. The pellets were resuspended in fresh buffer and incubated at 37°C for 30 min. Following incubation, the suspensions were centrifuged as before, the resulting pellets resuspended in 100 volumes of 50 mM Tris, pH 7.4 plus 2 mg/ml bovine serum and the suspensions combined. Each assay tube contained 0.5 ml of membrane suspension, 2 nM [³H]-DAMGO, in a total volume of 0.65 ml.

Imaging: Experiments were carried out on a Zeiss Axiovert Microscope equipped with a scanning stage, an incubation chamber, an ultra-high speed Lambda DG-4 wavelength switcher, and an Axiocam CCD camera (Zeiss, Gottingen, Germany) all controlled by Axiovision software 4.8. Cells were maintained during imaging at 37°C in a 5% CO₂ atmosphere. Filters used were; green excitation (480/20nm) green emission (530/20nm) and orange excitation (531/40nm), orange emission (593/40nm) and the dichroics used were FF495 and FF559 respectively (Semrock, Rochester, NY).

Results and Discussion

GFP-labeled MOR

We have optimized a signal sequence that allows for efficient extracellular expression of FP-fused GPCRs with short amino terminal tails. For GFP-MOR, the green fluorescent protein was fused to the N-terminal end of the receptor by preparation of a cloning vector (pN1), a 23 amino acid sequence was inserted immediately following the initiating methionine, a linker of 2 amino acids was used between the signal sequence and GFP, a second linker (Gly-Ser-Gly) was used between the GFP and the first amino acid of the opioid receptors. Expression of the vector in COS cells revealed a high number of cells expressing the fluorescent receptor at the cell surface. External presentation of the GFP was confirmed using antibodies raised against the Flag tag sequence (FADYKDDDDK) present on the sequence between the signal peptide and GFP (Figure 1).

To test whether the external GFP interfered with binding to the receptor we compared the binding affinity of DAMGO, a standard opioid ligand, at the N-terminal fused GFP-MOR compared to the receptor with the fluorescent protein fused to the C-terminal. The binding affinity of GFP-MOR ($K_i = 3.06$ nM), did not differ from that of the C-terminal fused receptor ($K_i = 2.96$ nM) as determined in a radioreceptor binding assay (Figure 2). Differences observed in the total bound values are due to expression of the GFP-MOR by transient transfection versus a stable clone cell line

Fig. 1. Surface expression in COS7 cells of opioid receptors (OPR) with GFP fused to the N-terminal of the receptor. Left panel: Green fluorescent intensity for GFP-OPR. Middle panel: Fluorescent intensity for red FP-labeled antibodies bound to flag-tag on GFP-OPR. Antibodies are bound to non-permeabilized fixed cells demonstrating the external expression of the GFP. Right panel: Merge of images demonstrating overlap of GFP and RFP fluorescence.

Fig. 2. Radioreceptor binding assay for GFP-MOR expressed in HEK293 cells. ³H-DAMGO was observed to bind to cells expressing MOR with similar affinities whether the fluorescent protein was fused to N- or C-terminal. Graphs represent the average $\pm$ sem of three replicates.

for MOR-YFP. We have successfully applied the *N*-terminal fusion method to all 3 opioid receptors (MOR, DOR, and KOR) and the orexin 2 receptor.

Use of Fluorescent labeled cyclic peptide for FRET.

We have previously designed, synthesized, and identified a fluorescent labeled MOR ligand from a mixture-based penta-cyclic peptide library [11]. Screening and de-convolution of a penta-cyclic peptide library identified a potent and selective MOR ligand (Figure 3) exhibiting a binding affinity of $K_i=14$ nM. We prepared analogues of this peptide in which the anthraniloyl group was replaced with Rhodamine, the binding affinity was not adversely affected by the addition of the fluorescent dye.

Fig. 3. Selective MOR ligand.

Fig. 4. Binding of Rhodamine labeled cyclic peptide to CHO cells. Cells transfected with OFP labeled MOR were exposed to 100 μ M peptide and images taken at 1 minute intervals for 20 mins. Filters used were; 559/34 for excitation; 607/36 for emission and a GFP Dichroic FF593.

To determine that cells expressing the *N*-terminal fusion protein were capable of binding the rhodamine labeled cyclic peptide we performed a time-lapsed imaging experiment in which we compared the increase in red fluorescence on the cell surface between cells transfected with the FP-MOR and non-transfected cells. There was a fivefold increase in the level of rhodamine fluorescence observed on the cells expressing the *N*-terminal GFP fusion (Figure 4). We then used a combination of the rhodamine labeled MOR ligand and the *N*-terminal fusion of GFP to the mu opioid receptor to determine if there was FRET between the ligand and the externally expressed GFP. FRET between GFP-labeled MOR and Rhodamine labeled cyclic peptide was demonstrated in HEK293 cells using live cell imaging, cells expressing the *N*-terminal GFP-MOR were labeled with the cyclic peptide for 30 minutes (images taken at 5 min intervals), then the fluorescent ligand was washed out and cells

Fig. 5. Binding of rhodamine-labeled MOR peptide demonstrates FRET with GFP-labeled MOR receptor. A. GFP labeled HEK293 cells. Demonstrate cell surface expression (708ms). B. Use of excitation at green wavelength (489/20nm) and emission at red wavelength (593/40nm) reveals FRET in all GFP expressing cells (1300ms Can). C. Excitation at rhodamine channels (531/40nm) and emission at red channel before addition of peptide demonstrates a small leak through for the brightest cell, but FRET values increase 5 fold upon addition of rhodamine labeled peptide (all exposures 1300ms). F. Comparison of Fret (cyan) and red channels (red) demonstrate the occurrence of efficient FRET correlates with the expression of MOR-GFP. Fret is observed in GFP labeled cells but minimal in non-transfected cells (NT, all exposures 1300 ms).

imaged for another 15 minutes (Figure 5). Images were taken using excitation and emission of for Green fluorescence and red fluorescence and excitation at green and emission at red wavelengths, FRET was observed between the ligand and receptor in transfected cells but not in non-transfected cells (Figure 5F).

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